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(71) **Applicant** *(for all designated States except US)*: AMYLOGENE HB [SE/SE]; c/o Svalöf Weibull AB, S-268 81 Svalöv (SE).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: EK, Bo [SE/SE]; Nyhagen, S-740 30 Björklinge (SE). KHOSNOODI, Jamshid [SE/SE]; Bandstolsvägen 3, 2 tr., S-756 48 Uppsala (SE). LARSSON, Clas-Tomas [SE/SE]; Flogstavägen 55 B II, S-752 73 Uppsala (SE). LARSSON, Håkan [SE/SE]; Hammarbygatan 58, S-753 24 Uppsala (SE). RASK, Lars [SE/SE]; Säves väg 14, S-752 63 Uppsala (SE).

(74) **Agent:** AWAPATENT AB, P.O. Box 5117, S-200 71 Malmö (SE).

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(54) **Title:** STARCH BRANCHING ENZYME II OF POTATO

(57) Abstract

The present invention relates to an amino acid sequence of second starch branching enzyme (SBE II) of potato and a fragment thereof as well as to the corresponding isolated DNA sequences. Furthermore, the invention relates to vectors comprising such an isolated DNA sequence, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch. The starch obtained will show a changed pattern of branching of amylopectin as well as a changed amylose/amylopectin ratio.

STARCH BRANCHING ENZYME II OF POTATO

The present invention relates to a novel starch branching enzyme of potato. More specifically, the present invention relates to an amino acid sequence of a second starch branching enzyme (SBE II) of potato and a fragment thereof as well as their corresponding DNA sequences. Furthermore, the invention relates to vectors comprising such DNA sequences, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch.

Starch is a complex mixture of different molecule forms differing in degree of polymerization and branching of the glucose chains. Starch consists of amylose and amylopectin, whereby the amylose consists of an essentially linear α-1,4-glucan and amylopectin consists of α-1,4-glucans connected to each other via α-1,6-linkages and, thus, forming a branched polyglucan. Thus, starch is not a uniform raw material.

Starch is synthesized via at least three enzymatic reactions in which ADP glucose phosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21) and starch branching enzyme (EC 2.4.1.18) are involved. Starch branching enzyme (SBE, also called Q-enzyme) is believed to have two different enzymatic activities. It catalyzes both the hydrolysis of α-1,4-glucosidic bonds and the formation of α-1,6-glucosidic bonds during synthesis of the branched component in starch, i.e. amylopectin.

Plant starch is a valuable source of renewable raw material used in, for example, the chemical industry (Visser and Jacobsen, 1993). However, the quality of the starch has to meet the demands of the processing industry wherein uniformity of structure is an important criterion. For industrial application there is a need of plants only containing amylose starch and plants only containing amylopectin starch, respectively.

fragments thereof, which after insertion into the genome of the plants cause changes in said branching degree and ratio in regenerated plants.

Within the scope of the present invention there is also included the amino acid sequence of SBE II and fragments thereof.

Also variants of the above DNA sequence resulting from the degeneracy of the genetic code are encompassed.

The novel DNA sequence encoding SBEII, comprising 3074 nucleotides, as well as the corresponding amino acid sequence comprising 878 amino acids, are shown in SEQ ID No. 1. One 1393 nucleotides long fragment of the above DNA sequence, corresponding to nucleotides 1007 to 2399 of the DNA sequence in SEQ ID No. 1, as well as the corresponding amino acid sequence comprising 464 amino acids, are shown in SEQ ID No. 2.

Furthermore, there are provided vectors comprising said isolated DNA-sequences and regulatory elements active in potato. The DNA sequences may be inserted in the sense or antisense (reversed) orientation in the vectors in relation to a promoter immediately upstream from the DNA sequence.

Also there is provided a process for the production of transgenic potatoes with a reduced degree of branching of amylopectin starch, comprising the following steps:
a) transfer and incorporation of a vector according to the invention into the genome of a potato cell, and
b) regeneration of intact, whole plants from the transformed cells.

Finally, the invention provides the use of said transgenic potatoes for the production of starch.

The invention will be described in more detail below in association with an experimental part and the accompanying drawings, in which:

Fig. 1 shows SDS polyacrylamide electrophoresis of proteins extracted from starch of normal potato (lane A)

dissolved in 50 mM Tris-HCl, pH 7.5. An aliquot of each preparation was analyzed by SDS poly-acrylamide gel electrophoresis according to Laemmli (1970)(Fig. 1). The proteins in the remaining portions of the preparations were concentrated by precipitation with trichloroacetic acid (10%) and the proteins were separated on an 8% SDS polyacrylamide gel Laemmli, (1970). The proteins in the gel were stained with Coomassie Brilliant Blue R-250 (0.2% in 20% methanol, 0.5% acetic acid, 79.5% H_2O).

In gel digestion and sequencing of peptides

The stained bands marked with arrows in Fig. 1 corresponding to an apparent molecular weight of about 100 kDa were excised and washed twice with 0.2M NH_4HCO_3 in 50% acetonitrile under continuous stirring at 35°C for 20 min. After each washing, the liquid was removed and the gel pieces were allowed to dry by evaporation in a fume hood. The completely dried gel pieces were then separately placed on parafilm and 2 µl of 0.2M NH_4CO_3, 0.02% Tween-20 were added. Modified trypsin (Promega, Madison, WI,USA)(0.25 µg in 2 µl) was sucked into the gel pieces whereafter 0.2M NH_4CO_3 was added in 5 µl portions until they had resumed their original sizes. The gel slices were further divided into three pieces and transferred to an Eppendorf tube. 0.2M NH_4CO_3 (200 µl) was added and the proteins contained in the gel pieces were digested over night at 37°C (Rosenfeld et al. 1992). After completed digestion, trifluoroacetic acid was added to 1% and the supernatants removed and saved. The gel pieces were further extracted twice with 60% acetonitrile, 0.1% trifluoroacetic acid (200 µl) under continuous shaking at 37°C for 20 min. The two supernatants from these extractions were combined with the first supernatant. The gel pieces were finally washed with 60% acetonitrile, 0.1% trifluoroacetic acid, 0.02% Tween-20 (200 µl). Also these supernatants were combined with the other supernatants and the volume was reduced to 50 µl by evaporation. The

Purification of mRNA from potato tuber, synthesis of cDNA and PCR amplification of a cDNA fragment corresponding to potato starch branching enzyme II.

Total RNA from mature potato tubers (*S. tuberosum* cv. Amanda) was isolated as described (Logemann et al. 1987). First strand cDNA was synthesized using 2 µg of total RNA and 60 pmol of oligo-dT_{30} as downstream primer. The primer was annealed to the polyA of the mRNA at 60°C for 5 min. The extension of the cDNA was performed according to the technical manual of the manufacturer using the Riboclone® cDNA Synthesis System M-MLV (H-)(Promega).

cDNA encoding the novel starch branching enzyme II according to the invention was amplified in a Perkin-Elmer GeneAmp® 9600 PCR thermocycler (Perkin-Elmer Cetus Instruments, CT, USA) using the two degenerate primers designed from the peptides 1 and 2 (see above) under the following conditions: 1 mM dNTP, 1 µM of each primer and an alicot of the cDNA described above in a total reaction volume of 20 µl with 1x AmpliTaq® buffer and 0,8 U AmpliTaq® (Perkin-Elmer Cetus). The cycling conditions were: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 15'), an unintended drop to 25°C, five cycles of 94°C for 20", 45°C for 1', ramp to 72°C for 1' and 72°C for 2', and 30 cycles of 94°C for 5", 45°C for 30", and 72°C for (2'+2" per cycle) and completed with 72°C for 10' prior to chilling to 4°C.

A sample of this reaction (0.1 µl) was reamplified using the cycling conditions: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 5'), five cycles of 94°C for 20'', 45°C for 1', and 72°C for 2', and 25 cycles of 94°C for 5'', 45°C for 30'', and 72°C for (2' + 2'' per cycle) and completed with 72°C for 10' prior to chilling to 4°C. After completion of the PCR amplification, the reaction was loaded on a 1.5% Seakem® agarose gel (FMC Bioproducts, Rockland, ME, USA). After electrophoresis and staining with ethidium bromide a major

branching enzyme I and about 80% identity to starch branching enzyme II from other plant species. The present inventors therefore denote the enzyme encoded by the new branching enzyme sequence potato starch branching enzyme II.

Transformation of potato plants

The isolated full length cDNA of potato starch branching enzyme II and other functionally active fragments in the range of 50-3 074 bp are cloned in reverse orientation behind promoters active in potato tubers. By the term "functionally active" is meant fragments that will affect the amylose/amylopectin ratio in potato starch. The DNA and amino acid sequence of SBE II according to the invention as well as one fragment of the DNA and corresponding amino acid sequence are shown in SEQ ID No. 1 and 2, respectively.

The promoters are selected from, for example, the patatin promoter, the promoter from the potato granule-bound starch synthase I gene or promoters isolated from potato starch branching enzymes I and II genes.

The constructs are cloned by techniques known in the art either in a binary Ti-plasmid vector suitable for transformation of potato mediated by *Agrobacterium tumefaciens*, or in a vector suitable for direct transformation using ballistic techniques or electroporation. It is realized that the sense (see below) and antisense constructs must contain all necessary regulatory elements.

Transgenic potato plants transcribe the inverse starch branching enzyme II construct specifically in tubers, leading to antisense inhibition of the enzyme. A reduction and changed pattern of the branching of amylopectin as well as a changed amylose/amylopectin ratio thereby occur in tuber starch.

The antisense construct for potato starch branching enzyme II is also used in combination with antisense

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GCA ATT GAC AAG TAT GAG GGT GGT TTG GAA GCT TTT TCT CGT GGT TAT      854
Ala Ile Asp Lys Tyr Glu Gly Gly Leu Glu Ala Phe Ser Arg Gly Tyr
    160                 165                 170

GAA AAA ATG GGT TTC ACT CGT AGT GCT ACA GGT ATC ACT TAC CGT GAG      902
Glu Lys Met Gly Phe Thr Arg Ser Ala Thr Gly Ile Thr Tyr Arg Glu
175                 180                 185                 190

TGG GCT CCT GGT GCC CAG TCA GCT GCC CTC ATT GGA GAT TTC AAC AAT      950
Trp Ala Pro Gly Ala Gln Ser Ala Ala Leu Ile Gly Asp Phe Asn Asn
                195                 200                 205

TGG GAC GCA AAT GCT GAC ATT ATG ACT CGG AAT GAA TTT GGT GTC TGG      998
Trp Asp Ala Asn Ala Asp Ile Met Thr Arg Asn Glu Phe Gly Val Trp
            210                 215                 220

GAG ATT TTT CTG CCA AAT AAT GTG GAT GGT TCT CCT GCA ATT CCT CAT     1046
Glu Ile Phe Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His
        225                 230                 235

GGG TCC AGA GTG AAG ATA CGT ATG GAC ACT CCA TCA GGT GTT AAG GAT     1094
Gly Ser Arg Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp
    240                 245                 250

TCC ATT CCT GCT TGG ATC AAC TAC TCT TTA CAG CTT CCT GAT GAA ATT     1142
Ser Ile Pro Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile
255                 260                 265                 270

CCA TAT AAT GGA ATA TAT TAT GAT CCA CCC GAA GAG GAG AGG TAT ATC     1190
Pro Tyr Asn Gly Ile Tyr Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile
                275                 280                 285

TTC CAA CAC CCA CGG CCA AAG AAA CCA AAG TCG CTG AGA ATA TAT GAA     1238
Phe Gln His Pro Arg Pro Lys Lys Pro Lys Ser Leu Arg Ile Tyr Glu
            290                 295                 300

TCT CAT ATT GGA ATG AGT AGT CCG GAG CCT AAA ATT AAC TCA TAC GTG     1286
Ser His Ile Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val
        305                 310                 315

AAT TTT AGA GAT GAA GTT CTT CCT CGC ATA AAA AAG CTT GGG TAC AAT     1334
Asn Phe Arg Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn
    320                 325                 330

GCG GTG CAA ATT ATG GCT ATT CAA GAG CAT TCT TAT TAT GCT AGT TTT     1382
Ala Val Gln Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe
335                 340                 345                 350

GGT TAT CAT GTC ACA AAT TTT TTN GCA CCA AGC AGC CGT TTT GGA ACN     1430
Gly Tyr His Val Thr Asn Phe Xaa Ala Pro Ser Ser Arg Phe Gly Thr
                355                 360                 365

CCC GAC GAC CTT AAG TCT TTG ATT GAT AAA GCT CAT GAG CTA GGA ATT     1478
Pro Asp Asp Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile
            370                 375                 380

GTT GTT CTC ATG GAC ATT GTT CAC AGC CAT GCA TCA AAT AAT ACT TTA     1526
Val Val Leu Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu
        385                 390                 395

GAT GGA CTG AAC ATG TTT GAC GGC ACA GAT AGT TGT TAC TTT CAC TCT     1574
Asp Gly Leu Asn Met Phe Asp Gly Thr Asp Ser Cys Tyr Phe His Ser
    400                 405                 410
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TAT GAT AAA TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA   2390
Tyr Asp Lys Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu
                675                 680                 685

AGA TAC CGT GGG TTG CAA GAA TTT GAC CGG GCT ATG CAG TAT CTT GAA   2438
Arg Tyr Arg Gly Leu Gln Glu Phe Asp Arg Ala Met Gln Tyr Leu Glu
            690                 695                 700

GAT AAA TAT GAG TTT ATG ACT TCA GAA CAC CAG TTC ATA TCA CGA AAG   2486
Asp Lys Tyr Glu Phe Met Thr Ser Glu His Gln Phe Ile Ser Arg Lys
        705                 710                 715

GAT GAA GGA GAT AGG ATG ATT GTA TTT GAA AAA GGA AAC CTA GTT TTT   2534
Asp Glu Gly Asp Arg Met Ile Val Phe Glu Lys Gly Asn Leu Val Phe
    720                 725                 730

GTC TTT AAT TTT CAC TGG ACA AAA AGC TAT TCA GAC TAT CGC ATA GGC   2582
Val Phe Asn Phe His Trp Thr Lys Ser Tyr Ser Asp Tyr Arg Ile Gly
735                 740                 745                 750

TGC CTG AAG CCT GGA AAA TAC AAG GTT GCC TTG GAC TCA GAT GAT CCA   2630
Cys Leu Lys Pro Gly Lys Tyr Lys Val Ala Leu Asp Ser Asp Asp Pro
                755                 760                 765

CTT TTT GGT GGC TTC GGG AGA ATT GAT CAT AAT GCC GAA TAT TTC ACC   2678
Leu Phe Gly Gly Phe Gly Arg Ile Asp His Asn Ala Glu Tyr Phe Thr
            770                 775                 780

TTT GAA GGA TGG TAT GAT GAT CGT CCT CGT TCA ATT ATG GTG TAT GCA   2721
Phe Glu Gly Trp Tyr Asp Asp Arg Pro Arg Ser Ile Met Val Tyr Ala
        785                 790                 795

CCT AGT AGA ACA GCA GTG GTC TAT GCA CTA GTA GAC AAA GAA GAA GAA   2774
Pro Ser Arg Thr Ala Val Val Tyr Ala Leu Val Asp Lys Glu Glu Glu
    800                 805                 810

GAA GAA GAA GAA GTA GCA GTA GTA GAA GAA GTA GTA GTA GAA GAA GAA   2822
Glu Glu Glu Glu Val Ala Val Val Glu Glu Val Val Val Glu Glu Glu
815                 820                 825                 830

TGA ACGAA CTTGTGATCG CGTTGAAAGA TTTGAAGGCT ACATAGAGCT TCTTGACGTA   2880
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TCTGGCAATA TTGCATCAGT CTTGGCGGAA TTTCATGTGA CAAAAGGTTT GCAATTCTTT   2940
CCACTATTAG TAGTGCAACG ATATACGCAG AGATGAAGTG CTGCACAAAC ATATGTAAAA   3000
TCGATGAATT TATGTCGAAT GCTGGGACGG GCTTCAGCAG GTTTTGCTTA GTGAGTTCTG   3060
TAAATTGTCA TCTC                                                     3074
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TTC AAA TTT GAT GGA TTT AGA TTT GAT GGT GTG ACA TCA ATG ATG TAT     721
Phe Lys Phe Asp Gly Phe Arg Phe Asp Gly Val Thr Ser Met Met Tyr
225                 230                 235                 240

ACT CAC CAC GGA TTA TCG GTG GGA TTC ACT GGG AAC TAC GAG GAA TAC     769
Thr His His Gly Leu Ser Val Gly Phe Thr Gly Asn Tyr Glu Glu Tyr
                245                 250                 255

TTT GGA CTC GCA ACT GAT GTG GAT GCT GTT GTG TAT CTG ATG CTG GTC     812
Phe Gly Leu Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met Leu Val
            260                 265                 270

AAC GAT CTT ATT CAT GGG CTT TTC CCA GAT GCA ATT ACC ATT GGT GAA     865
Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr Ile Gly Glu
        275                 280                 285

GAT GTT AGC GGA ATG CCG ACA TTT TNT ATT CCC GTT CAA GAT GGG GGT     913
Asp Val Ser Gly Met Pro Thr Phe Xaa Ile Pro Val Gln Asp Gly Gly
    290                 295                 300

GTT GGC TTT GAC TAT CGG CTG CAT ATG GCA ATT GCT GAT AAA TGG ATT     961
Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp Lys Trp Ile
305                 310                 315                 320

GAG TTG CTC AAG AAA CGG GAT GAG GAT TGG AGA GTG GGT GAT ATT GTT    1019
Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly Asp Ile Val
                325                 330                 335

CAT ACA CTG ACA AAT AGA AGA TGG TCG GAA AAG TGT GTT TCA TAC GCT    1057
His Thr Leu Thr Asn Arg Arg Trp Ser Glu Lys Cys Val Ser Tyr Ala
            340                 345                 350

GAA AGT CAT GAT CAA GCT CTA GTC GGT GAT AAA ACT ATA GCA TTC TGG    1105
Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile Ala Phe Trp
        355                 360                 365

CTG ATG GAC AAG GAT ATG TAT GAT TTT ATG GCT CTG GAT AGA CCN TCA    1153
Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp Arg Pro Ser
    370                 375                 380

ACA TCA TTA ATA GAT CGT GGG ATA GCA TTG CAC AAG ATG ATT AGG CTT    1201
Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met Ile Arg Leu
385                 390                 395                 400

GTA ACT ATG GGA TTA GGA GGA GAA GGG TAC CTA AAT TTC ATG GGA AAT    1249
Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe Met Gly Asn
                405                 410                 415

GAA TTC GGC CAC CCT GAG TGG ATT GAT TTC CCT AGG GCT GAA CAA CAC    1297
Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala Glu Gln His
            420                 425                 430

CTC TCT GAT GGC TCA GTA ATT CCC GGA AAC CAA TTC AGT TAT GAT AAA    1345
Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Ser Tyr Asp Lys
        435                 440                 445

TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA AGA TAC CGT    1393
Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu Arg Tyr Arg
    450                 455                 460
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starch, c h a r a c t e r i z e d in that it comprises the following steps:

a) transfer and incorporation of a vector according to claim 8 into the genome of a potato cell, and

b) regeneration of intact, whole plants from the transformed cells.

11. A process according to claim 10, wherein the vector also comprises an antisense construct of starch branching enzyme I (SBE I).

12. A process according to claims 10 or 11, wherein the vector also comprises an antisense construct of potato granule bound starch synthase II.

13. A process according to one or more of claims 10-12, wherein the vector also comprises an antisense construct of potato soluble starch synthases II and III.

14. A process according to one or more of claims 10-13, wherein the vector also comprises an antisense construct of potato starch disproportionating enzyme (D-enzyme).

15. A process according to one or more of claims 10-14, wherein the vector also comprises an antisense construct of potato starch debranching enzyme.

16. A transgenic potato obtainable by the process according to any one of claims 9-15.

17. Use of transgenic potatoes according to claim 16 for the production of starch.

FIG. 2

Peptide 1. EFGVWEIFLPN

Peptide 2. HGLQEFDRA

Peptide 3. ENDGIAAKADE

Peptide 4. YEIDPEI/LTN

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9504826	16/02/95	AU-A- 7535294	28/02/95
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		CA-A- 2169174	16/02/95
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		IL-D- 110583	00/00/00
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		SE-A- 9004095	01/06/92
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(74) Agent: AWAPATENT AB; P.O. Box 5117, S-200 71 Malmö (SE).

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(54) Title: STARCH BRANCHING ENZYME II OF POTATO

(57) Abstract

The present invention relates to an amino acid sequence of second starch branching enzyme (SBE II) of potato and a fragment thereof as well as to the corresponding isolated DNA sequences. Furthermore, the invention relates to vectors comprising such an isolated DNA sequence, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch. The starch obtained will show a changed pattern of branching of amylopectin as well as a changed amylose/amylopectin ratio.

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9634968 A2 (NATIONAL STARCH AND CHEMICAL INVESTMENT HOLDING CORPORATION), 7 November 1996 (07.11.96) --	1-17
X	WO 9504826 A1 (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 16 February 1995 (16.02.95), see abstract and claim 23 --	1-17
X	WO 9214827 A1 (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 3 Sept 1992 (03.09.92), see page 5, line 1-7 and examples --	1-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 March 1998	01 -04- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Stösteen Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/98

International application No.
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